Structure−Activity Relationship for Noncoplanar Polychlorinated Biphenyl Congeners toward the Ryanodine Receptor-Ca^{2+} Channel Complex Type 1 (RyR1)

Isaac N. Pessah,*[,†] Larry G. Hansen,[‡] Timothy E. Albertson,[§] C. Edwin Garner,[||,⊥] Tram Anh Ta,[†] Zung Do,[†] Kyung Ho Kim,[†] and Patty W. Wong[†,#]

Department of VM:Molecular Biosciences and Center for Children's Environmental Health, and Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, School of Medicine, University of California, Davis, California 95616, Department of Veterinary Biosciences, College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61802, and Laboratory of Pharmacology and Chemistry, Environmental Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

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Ryanodine receptor isoforms are expressed in both excitable and nonexcitable tissues where they form microsomal Ca^{2+} release channels broadly involved in shaping cellular signaling. In this report, we provide a detailed structure−activity relationship (SAR) for polychlorinated biphenyl (PCB) congeners and metabolites necessary for enhancing ryanodine receptor type 1 (RyR1) activity using [^{3}H]ryanodine ([^{3}H]Ry) binding analysis. The 2,3,6-Cl PCB configuration is most important for optimal recognition by the RyR1 complex and/or critical for sensitizing its activation. Para substitution(s) diminishes the activity with *para*-chloro having a higher potency than the corresponding *para*-hydroxy derivative. The addition of a more bulky *para*-methyl-sulfonyl group eliminates the activity toward RyR1, supporting the importance of the para positions in binding RyR1. The requirement for an intact major T cell immunophilin FKBP12−RyR1 complex was observed with each of 12 active PCB congeners indicating a common mechanism requiring an immunophilin-regulated Ca^{2+} release channel. An excellent correlation between the relative potencies for doubling [^{3}H]Ry binding and the corresponding initial rates of PCB-induced Ca^{2+} efflux indicates that [^{3}H]Ry binding analysis provides a measure of dysregulation of microsomal Ca^{2+} transport. The SAR for activating RyR1 is consistent with those previously reported in several in vivo and in vitro studies, suggesting that a common mechanism may contribute to the toxicity of noncoplanar PCBs. A practical application of the receptor-based screen developed here with RyR1 is that it provides a quantitative SAR that may be useful in predicting biological activity and risk of mixtures containing noncoplanar PCB congeners that have low or a lack of aryl hydrocarbon receptor activity.

Introduction

Polychlorinated biphenyls (PCBs)[1] along with polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are halogenated aromatic hydrocarbons (HAHs). In the United States, PCBs were synthesized and marketed as Aroclor mixtures, which were widely used in several industries. Together, the improper disposal, the high lipophilicity, and the extreme chemical resistance of these chemicals led to worldwide contamination and accumulation in biota (*1*−*3*). Because of their diverse structures, differences in ecosystem partitioning, and variable metabolic degradations, complex mixtures of PCB congeners in biological samples with proportions unique to sources and species have been found (*1*). Attention has been directed toward ortho-poor PCB congeners (i.e., tetra- through octachlorobiphenyls with single or no *ortho*-chlorine atoms) because they can assume a coplanar configuration and interact with the aryl hydrocarbon receptor (AhR), mimicking the action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*2*−*4*). However, ortho-rich PCBs (congeners with 2−4 *ortho*-chlorine atoms) are more prevalent in former commercial mixtures, environmental reservoirs, and human samples (*1*, *5*−*7*). The relative abundance of ortho-rich PCBs is further noted in air because of greater volatility (*8*−*11*). In fish from contaminated temperate waters, *ortho*-PCBs are enriched due to differential inducible metabolism (*12*). Indeed, there are elevated levels of ortho-substituted PCB congeners found in both the tissues of Great Lakes fish and the serum of humans consuming these fish (*7*, *13*−*15*).

* To whom correspondence should be addressed. Tel: 530-752-6696. Fax: 530-752-4698. E-mail: inpessah@ucdavis.edu.

[†] Department of VM:Molecular Biosciences and Center for Children's Environmental Health, University of California.

[‡] University of Illinois.

[§] School of Medicine, University of California.

[||] National Institute of Environmental Health Sciences.

[⊥] Current address: Department of Drug Metabolism and Pharmacokinetics, Schering-Plough Research Institute, P.O. Box 32, 144 Route 94, Lafayette, NJ 07848-0032.

[#] Current address: Department of Toxic Substances Control, 1001 I Street, Sacramento, CA 95814.

[1] Abbreviations: Ah, aryl hydrocarbon; Ah (H), aryl hydrocarbon hydroxylase; CICR, Ca^{2+}-induced Ca^{2+} release; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration that causes enhancement to 50% of the maximum activity; EROD, ethoxyresorufin *o*-deethylase; FKBP12/RyR, immunophilin FKBP12/ryanodine receptor complex; HAHs, halogenated aromatic hydrocarbons; [^{3}H]Ry, tritium-labeled ryanodine; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; PCBs, polychlorinated biphenyls; PCDFs, dibenzofurans; PCDDs, polychlorinated dibenzo-*p*-dioxins; PC12, rat pheochromocytoma cells; RyR1, ryanodine receptor type 1; RyR, all three isoforms of ryanodine receptor; SR/ER, sarcoplasmic or endoplasmic reticulum; SARs, structure−activity relationships; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dibenzodioxin; TEFs, (toxic) TCDD equivalence factors; TEQ, (toxic) TCDD equivalence; TNP-LPS, trinitrophenyl-lipopolysaccharide.

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Finally, anaerobic meta/para dechlorination of highly chlorinated PCB congeners in sediments, and hence propagation along the food chain, also may contribute to enriched levels of ortho-substituted PCB congeners in human serum as compared to that in the Aroclor mixtures (*15*, *16*).

Many of the ortho-poor PCBs have high affinities for the AhR and can elicit responses despite generally low environmental occurrences (*4*). The ortho-rich PCBs and metabolites of both ortho-rich and ortho-poor PCBs have a number of actions independent of the AhR. Endocrine effects include weak estrogenicity, disruption of the hypothalamo−pituitary−thyroid axis, enhanced insulin secretion, and increased release of arachidonic acid (*17*−*24*). Epidemiological studies have indicated that perinatal exposures to PCBs are correlated with cognitive deficits in children (*3*, *25*). Schantz and co-workers have reported on the impact of PCBs on intellectual functioning in both children and older adults; exposure to PCBs, but not dichlorodiphenyl dichloroethene, was associated with memory and learning impairments, consistent with the previous reports on in utero exposure of PCBs and cognitive deficits in children (*26*, *27*). Significantly higher levels of ortho-substituted PCBs have been reported in the caudate nucleus of patients with Parkinson's disease (*28*).

Mechanistic studies on the neurotoxicity of ortho-substituted PCB congeners have implicated these chemicals in neurotoxic responses. Structure−activity relationships (SARs) between selected PCB congeners and cellular catecholamine levels in neurons have been examined both in vitro using rat pheochromocytoma cells (PC12) and in vivo using nonhuman primates (*29*, *30*). Further studies have demonstrated that disruption of Ca^{2+} signaling may be the underlying mechanism for certain non-Ah receptor-mediated responses to ortho-substituted PCB congeners (*31*, *32*).

Data identifying more selective interactions between ortho-substituted PCBs and microsomal Ca^{2+} release channels suggest that they may explain in a large part many of the cellular toxicities described for noncoplanar PCBs (*33*−*39*). These channels include both inositol 1,4,5-trisphosphate receptors (IP_3Rs) (*40*) and genetically related ryanodine receptors (RyRs) and may both be receptor targets of noncoplanar PCBs (*39*). Specifically noncoplanar, but not coplanar, PCBs have been shown to directly enhance the sensitivity of RyRs to activation by endogenous ligands in a manner requiring the major T cell immunophilin FKBP12, which forms a physical association with RyR (FKBP12−RyR complex) (*36*, *38*, *41*, *42*). IP_3Rs and RyRs are likely relevant toxicological targets of noncoplanar PCBs given their diverse contributions toward regulating the release of Ca^{2+} from sarcoplasmic and endoplasmic reticulum (SR/ER) and integrating cellular signaling events in excitable tissues (*43*−*45*). Moreover, RyRs have been shown to be involved in chemically induced oxidative stress (*46*) and contribute molecular disorders of skeletal (*47*) and cardiac muscle (*48*).

In this report, we provide a detailed quantitative SAR showing that PCB congeners and metabolites alter ryanodine receptor type 1 (RyR1) activity. The SAR for PCB-sensitized RyR1 is consistent with SARs previously reported for endocrine and neurochemical end points of toxicity. Considering that the three RyR isoforms are broadly expressed in both excitable and nonexcitable tissues, alterations in RyR function may represent a common mechanism whereby ortho-rich PCBs may alter neurological and endocrine function. A practical application of the receptor-based screen developed here with RyR1 is that it provides a quantitative SAR that may be useful in predicting biological activity and risk of environmental/food chain mixtures containing differing proportions of noncoplanar PCB congeners that have low or lack AhR activity.

Experimental Procedures

PCB Stocks. Neat PCB congeners were purchased from Ultra Scientific (North Kingstown, RI) and Accu-Standards (New Haven, CT). Methyl-sulfonyl-PCB metabolites were generously provided by Christina Larsson and Äke Bergman (Stockholm University). All reagents used in the synthesis of the hydroxy-PCB metabolites were purchased from Aldrich Chemical Co. (Milwaukee, WI). Stocks of each PCB congener or metabolite were prepared by dissolving the neat chemicals in anhydrous dimethyl sulfoxide (DMSO). Rapamycin was purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO). All other chemicals were purchased commercially with the highest purity available.

Synthesis of Hydroxylated PCBs. The 2,3,6,2′,3′,6′-hexachloro-4-biphenylol (4-OH,PCB136); 2,4,6-trichloro-4′-biphenylol (4′-OH,-PCB30); 2,4,6-trichloro-3′,4′-biphenyldiol (3′,4′-di-OH,PCB30); 2,5-dichloro-2′,3′-biphenyldiol (2′,3′-di-OH,PCB9); 2,5-dichloro-3′,4′-biphenyldiol (3′,4′-di-OH,PCB9); 2,5-dichloro-3′-biphenylol (3′-OH,PCB9); and 2,5-dichloro-4′-biphenylol (4′-OH,PCB9) were synthesized at NIEHS (RTP, NC) as described previously (*21*). Briefly, mono- or dimethoxy anilines were coupled with chlorinated benzenes to yield the chlorinated mono- or dimethoxybiphenyls. The phenols or catechols were then produced by demethylation with boron tribromide. Structural identification was determined by NMR and GC/MS. All test compounds were determined to be greater than 95% pure by TLC and GC.

Environmental PCB Mixtures. The environmental samples were collected from the Sangamo Electric Co. manufacturing waste landfill, a National Priorities List Landfill on the Crab Orchard National Wildlife Refuge in Southern Illinois. The air sample was a composite of 18∼24 h, high-volume, filtered (vapor phase) collections. The "dust" consisted of superficial dust and debris directly under the air collection, while the "soil" was the deeper layers under the dust collection. The collection and extraction procedures were detailed in describing the high PCB content (*49*), the high proportion of PCDFs because of the practice of open burning (*50*), and some biological actions of the extracts (*24*, *51*). A comparative summary of the characteristics of the three environmental extracts is presented along with the discussion of the results.

Membrane Preparations. Microsomal membrane vesicles enriched in RyR1 were isolated from fast-twitch (white) skeletal muscles obtained from the back of male New Zealand White rabbits, as previously described (*52*, *53*). Briefly, muscle was ground and homogenized in ice-cold homogenization buffer consisting of 5 mM imidazole-HCl, pH 7.4, 0.3 M sucrose, 10 μg/mL leupeptin, and 100 μM phenylmethylsulfonyl fluoride. The junctional SR fraction was then isolated by differential centrifugation and further purified by discontinuous sucrose gradient.

Tritium-Labeled Ryanodine ([^{3}H]Ry) Binding Assay. Specific binding of [^{3}H]Ry to isolated skeletal microsomes was performed as previously reported (*38*), with slight modifications. Briefly, The ability of selected PCB congeners to dose-dependently alter [^{3}H]Ry binding to isolated skeletal microsomes was examined by measuring the specific binding of 1 nM [^{3}H]Ry to 12 μg of microsomal protein in the presence of 10 nM to 100 μM PCB in assay buffer consisting of 140 mM KCl, 15 mM NaCl, 20 mM HEPES, 10% sucrose, and 50 μM $CaCl_2$, pH 7.4, with a final volume of 500 μL. The control binding level in each experiment was determined by the addition of an equivalent volume of DMSO (solvent vesicle for PCBs) in the assay. Nonspecific binding was obtained by the addition of 1000-fold excess of unlabeled ryanodine in the assay.

Macroscopic Ca^{2+} Flux Measurement. Net Ca^{2+} efflux from isolated skeletal microsomes was measured with the metallochromic Ca^{2+} sensitive dye antipyrylazo III with the use of a diode array spectrophotometer (model 8542 or 8543, Agilent, Palo Alto, CA), as reported previously (*38*). Briefly, the Ca^{2+} transport buffer

consisted of 18.5 mM K-MOPS, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM antipyrylazo III, 1 mM Mg-ATP, 20 mg/mL creatine phosphokinase, 5 mM phosphocreatine, and 40 μg of skeletal microsome with a final volume of 1.2 mL. Active Ca^{2+} loading to the vesicles was performed by several additions of 20 nmol of Ca^{2+} bolus until the uptake capacity was reached. Ca^{2+} release was then induced by the addition of 7.5 μM PCB. The net Ca^{2+} efflux induced by solvent was determined by the addition of an equivalent amount of DMSO in the same experiment.

Data Analysis. With the more potent and efficacious PCB congeners, data across the entire dose−response curve were fit to a sigmoidal equation with Origin computer software (Microcal Software, Inc., Northampton, MA) to calculate three parameters: (i) the concentration of PCB needed to double basal receptor activity (EC_{2x}), (ii) the concentration needed to obtain 50% of the maximum receptor activity (EC_{50}), and (iii) the maximum receptor activation (B_{max}). The initial rate of Ca^{2+} release induced by selected PCBs was determined by linear regression analysis of Ca^{2+} release during the initial 30 s following the addition of PCB. The Ca^{2+} release rate data were calibrated by bolus addition of standard Ca^{2+} at the end of each assay. The net PCB-induced Ca^{2+} efflux rate was obtained by subtracting the residue change in Ca^{2+} upon addition of equivalent DMSO in the control experiment. Data were then normalized to that of the PCB95, the most active congener in both the [^{3}H]Ry binding assay and the active Ca^{2+} transport assay.

Results and Discussion

Stringent SAR for Noncoplanar PCBs toward Sensitizing RyR1 Activity. Equilibrium binding assays were performed to elucidate the SAR of 35 selected PCB congeners, eight hydroxylated PCB metabolites, and four methyl-sulfonyl-PCB metabolites on activation of RyR1. The radioligand [^{3}H]Ry binds to RyR1 with high selectivity and specificity. More importantly, [^{3}H]Ry binds with high affinity only to an activated conformation of RyR1 (*52*). By taking advantage of this conformational sensitive property of [^{3}H]Ry binding, we have established the dose−response relationship for selected PCBs (congeners and metabolites) toward sensitizing the activation of RyR1. For most of the PCB congeners tested, full dose−response curves were obtained and the calculated EC_{50} (a measure of potency) and B_{max} (a measure of intrinsic efficacy) values were calculated. The concentration of each PCB required to enhance the activity of RyR1 channels two times (EC_{2x}; $P < 0.05$) the control specific binding level was also calculated. The EC_{2x} parameter was found highly predictive of PCB-induced Ca^{2+} release rates from isolated microsomes (see below) and permitted inclusion of a few congeners that exhibited low intrinsic efficacy toward enhancing [^{3}H]Ry binding in the analysis of structure−activity. Table 1 summarizes the EC_{2x}, EC_{50}, and B_{max} values for the PCBs tested in the present study. Among the active PCB congeners studied, EC_{50} and B_{max} varied by greater than 10- and 5-fold, respectively. Coplanar PCBs and metabolites were inactive toward RyR1. For example, PCB126, one of the most potent PCB congeners toward the aryl hydrocarbon hydroxylase [Ah (H)] receptor, lacked activity toward RyR1 at its solubility limits (Table 1). In general, PCBs lacking at least one ortho substitution were inactive toward RyR1, regardless of the degree of chlorination. Figure 1 shows a ranking of the 28 most active congeners tested based on the concentration required to double the RyR1 activity.

Several of the PCB congeners tested are environmentally prevalent (Table 1). Among those, PCB18, -41, -52, -95, -101, -110, -149, and -187 were highly efficacious, enhancing the binding of [^{3}H]Ry from a basal level of $\leq$0.87 to a maximum of $\geq$4.0 pmol/mg, respectively. Of the 35 PCB congeners tested, 14 structures induce a doubling (EC_{2x}) of RyR1 activity at a concentration below 1 μM (Figure 1). Of these, PCB95 and 2,2′,3,3′,6,6′-hexachlorobiphenyl (PCB136) possessed the lowest EC_{50} values of $<$0.5 μM and EC_{2x} values of $\sim$0.25 μM (Figure 2). Both PCBs share a similar symmetry of chlorine substitutions on each of the phenyl rings (2,3,6 and 2′,3′,6′ for PCB136 vs 2,3,6 and 2′,5′ for PCB95). The 2,5- and 6,3-positions are both ortho,meta and could be read the same, although incorrect by nomenclature rules. Thus, the 2′,5′-Cl configuration of PCB95 is equivalent to the 3′,6′-Cl configuration of PCB136 (i.e., 2,3,6,2′,5′ vs 2,3,6,3′,6′) if the torsion angle between the two phenyl rings is 90°. Recently, Lehmler and colleagues determined that the crystal structure of PCB84 in solid state has a dihedral angle of 81° and a calculated dihedral angle of 90° (*54*). On the basis of these results, PCB95 and -136 are both likely to have dihedral angles of 90° in solution. As a result, PCB95 is a partial structure of PCB136 with the absence of an *ortho*-Cl substitution on the π-ring. These results suggest that the 2,3,6-Cl configuration on one ring with ortho, meta on the other make essential contributions for optimal recognition of the RyR1 complex and for sensitizing channel activation. The next most potent congeners having EC_{2x} values between 0.3 and 0.45 consisted of PCB84, -96, -149, and -176, which all satisfied these criteria. The environmentally important, but readily metabolized, PCB52 is also quite potent (Table 1), but it lacks the 2,3,6-substitution pattern (the 2,5,2′,5′ is equivalent to 3,6,3′6′). Likewise, the highly relevant PCB170, -101, -180, -18, -110, and -153 double RyR1 binding at similar, but slightly higher, concentrations and have only two ortho substitutions. The slightly more potent congeners (PCB151, -183, and -187) satisfy the tri-ortho substitution preference.

The SAR of PCBs toward RyR1 identified in the present study is consistent with previous results on the activity of noncoplanar congeners toward cultured neurons and from in vivo studies with rodents and nonhuman primates. For example, exposure of the nonhuman primate, *Macaca nemestrina*, to Aroclor 1016 produced decreased dopamine concentrations in the caudate, putamen, substantia nigra, and hypothalamus and was associated with detectable levels of only three ortho-substituted nonplanar PCB congeners (PCB28, -47, and -52) (*29*). In vitro testing demonstrated that these three congeners also reduced cellular dopamine concentrations while planar, dioxin-like congeners, e.g., PCB77 (3,4,3′,4′) and PCB126 (3,4,5,3′,4′), did not. In a more recent study, PCB47 was identified as a potent inducer of Ca^{2+}-dependent apoptosis in neurons cultured from rat hippocampus, whereas coplanar PCB77 had no activity (*33*). The proapoptotic activity of PCB47 (2,4,2′,4′) could be largely prevented in the presence of FLA-365, a blocker of RyRs (*55*), but not by blockers of other types of Ca^{2+} channels (*33*). Using [^{3}H]PDBu binding as a measure of PKC translocation in cerebellar granule cell cultures, Tilson and co-workers (*32*) studied the potency of 24 congeners and found that di- and tri-ortho congeners were the most potent whereas coplanar congeners were inactive. Collectively, results on PCB SARs indicate that the activity toward RyR1 (this study) and the two additional isoforms RyR2 (*38*) and RyR3 provides a valuable mechanistic basis for predicting the efficacy of noncoplanar PCB congeners/metabolites toward dysregulation of cellular Ca^{2+} signaling and downstream cellular dysfunctions observed both in vitro and in vivo. A recent analysis of complex PCB mixtures from Fox River walleye pike showed that the mixture had a relatively low AhR activity but a high ryanodine receptor activity and was predictive of the high composition of ortho-substituted PCBs (e.g., $>$5% PCB95 + PCB66) (*56*). We therefore propose that the relative potency of PCBs toward

Table 1. Relative Potencies and Efficacies of PCB Congeners Toward Activation of RyR1[a]

BZ no.[b]	Cl⁻ substitutions	EC_{2x} (μM)[c]	EC_{50} (μM)[c]	B_{max} (pmol mg^{-1})[d]
PCB4 ($n = 4$)	2, 2′	2.18 ± 0.27	5.61 ± 0.53	3.9 ± 0.33
PCB9 ($n = 4$)	2, 5	4.09 ± 2.03	0.98 ± 0.32	2.9 ± 0.28
PCB18 ($n = 6$)	2, 5, 2′	1.05 ± 0.09	2.81 ± 0.07	5.8 ± 0.69
PCB24 ($n = 4$)	2, 3, 6	2.98 ± 0.32	3.53 ± 0.30	3.0 ± 0.49
PCB26 ($n = 4$)	2, 5, 3′	3.53 ± 0.32	3.87 ± 0.37	3.5 ± 0.66
PCB27 ($n = 4$)	2, 6, 3′	1.51 ± 0.15	3.26 ± 0.18	5.3 ± 0.08
PCB30 ($n = 4$)	2, 4, 6	2.62 ± 0.79	10.92 ± 1.7	2.9 ± 0.10
PCB41 ($n = 5$)	2, 3, 4, 2′	1.51 ± 0.38	2.58 ± 0.10	4.7 ± 1.9
PCB49 ($n = 4$)	2, 4, 2′, 5′	1.82 ± 0.13	2.17 ± 0.44	3.4 ± 0.07
PCB52 ($n = 4$)	2, 5, 2′, 5′	0.49 ± 0.08	1.25 ± 0.24	4.0 ± 0.22
PCB66 ($n = 4$)	2, 4, 3′, 4′	2.38 ± 0.14	3.10 ± 0.40	1.7 ± 0.06
PCB70 ($n = 4$)	2, 5, 3′, 4′	3.10 ± 0.80	2.20 ± 0.16	2.2 ± 0.16
PCB75 ($n = 4$)	2, 4, 6, 4′	inactive	inactive	inactive
PCB84 ($n = 6$)	2, 3, 6, 2′, 3′	0.42 ± 0.7	1.80 ± 0.25	6.2 ± 0.20
PCB95 ($n = 11$)	2, 3, 6, 2′, 5′	0.22 ± 0.03	0.49 ± 0.15	7.5 ± 0.33
PCB96 ($n = 4$)	2, 3, 6, 2′, 6′	0.44 ± 0.08	0.77 ± 0.06	5.9 ± 0.01
PCB101 ($n = 5$)	2, 4, 5, 2′, 5′	0.86 ± 0.06	1.90 ± 0.32.	3.9 ± 0.06
PCB110 ($n = 6$)	2, 3, 6, 3′, 4′	1.13 ± 0.34	1.70 ± 0.22	4.6 ± 0.12
PCB111 ($n = 4$)	2, 3, 5, 3′, 5′	4.83 ± 1.50	ASL[e]	ASL[e]
PCB123 ($n = 4$)	2, 4, 3′, 4′, 5′	inactive	inactive	inactive
PCB126 ($n = 4$)	3,4,5,3′,4′	inactive	inactive	inactive
PCB132 ($n = 4$)	2, 3, 4, 2′, 3′, 6′	0.91 ± 0.04	1.80 ± 0.20	3.8 ± 0.46
PCB136 ($n = 4$)	2, 3, 6, 2′, 3′, 6′	0.23 ± 0.07	0.48 ± 0.09	4.5 ± 0.20
PCB138 ($n = 5$)	2, 3, 4, 2′, 4′, 5′	2.61 ± 0.59	4.30 ± 1.10	2.5 ± 0.23
PCB149 ($n = 5$)	2, 3, 6, 2′, 4′, 5′	0.33 ± 0.02	0.70 ± 0.03	7.7 ± 0.14
PCB151 ($n = 5$)	2, 3, 5, 6, 2′, 5′	0.50 ± 0.05	1.54 ± 0.34	5.4 ± 0.39
PCB153 ($n = 5$)	2, 4, 5, 2′, 4′, 5′	1.21 ± 0.15	2.86 ± 0.39	3.3 ± 0.51
PCB157 ($n = 4$)	2, 3, 4, 3′, 4′, 5′	inactive	inactive	inactive
PCB159 ($n = 3$)	2, 3, 4, 5, 3′, 5′	1.95 ± 0.41	>25	ASL[e]
PCB163 ($n = 5$)	2, 3, 5, 6, 3′, 4′	1.44 ± 0.18	1.92 ± 0.09	1.3 ± 0.50
PCB170 ($n = 5$)	2, 3, 4, 5, 2′, 3′, 4′	0.73 ± 0.12	1.51 ± 0.24	2.5 ± 0.27
PCB176 ($n = 4$)	2, 3, 4, 6, 2′, 3′, 6′	0.38 ± 0.05	0.82 ± 0.19	4.5 ± 1.03
PCB180 ($n = 5$)	2, 3, 4, 5, 2′, 4′, 5′	0.96 ± 0.22	2.62 ± 0.28	3.1 ± 0.05
PCB183 ($n = 5$)	2, 3, 4, 6, 2′, 4′, 5′	0.55 ± 0.07	1.31 ± 0.30	2.5 ± 0.46
PCB187 ($n = 5$)	2, 3, 5, 6, 2′, 4′, 5′	0.58 ± 0.06	1.83 ± 0.22	4.2 ± 0.33
4-OH,PCB136 ($n = 6$)	4-OH, 2, 3, 6, 2′, 3′, 6′	1.60 ± 0.26	ASL[e]	ASL[e]
4′-OH,PCB30 ($n = 6$)	4′-OH, 2, 4, 6	0.32 ± 0.04	0.75 ± 0.08	5.4 ± 0.22
3′,4′-di-OH,PCB30 ($n = 6$)	3′, 4′-di-OH, 2, 4, 6	13.06 ± 1.72	28.3 ± 1.9	2.7 ± 0.91
2′,3′-di-OH,PCB9 ($n = 6$)	2′, 3′-di-OH, 2, 5	15.77 ± 3.15	>25	ASL[e]
3′,4′-di-OH,PCB9 ($n = 6$)	3′, 4′-di-OH, 2, 5	ASL[e]		
3′-OH,PCB9 ($n = 6$)	3′-OH, 2, 5	1.69 ± 0.14	3.50 ± 0.21	6.2 ± 0.9
4′-OH,PCB9 ($n = 6$)	4′-OH, 2, 5	1.38 ± 0.12	2.25 ± 0.11	5.25 ± 0.34
4-CH_3SO_2-PCB95	3-CH_3SO_2, 2,3,6,2′,5′	inactive	inactive	inactive
4′-CH_3SO_2-PCB101	4′-CH_3SO_2, 2, 4, 5, 2′, 5′	inactive	inactive	inactive
3′-CH_3SO_2-PCB149	3′-CH_3SO_2, 2, 3, 6, 2′, 4′, 5′	inactive	inactive	inactive
4-CH_3SO_2-PCB149	4-CH_3SO_2, 2, 3, 6, 2′, 4′, 5′	inactive	inactive	inactive

[a] The potency and efficacy of select PCB congeners toward enhancing [^{3}H]Ry binding were analyzed as described in the Experimental Procedures. [b] BZ no. represents the Ballschmitter number used to identify each specific congener. [c] Data show averages and standard errors for [PCB] needed to enhance specific [^{3}H]Ry binding 2-fold (2×) and half of maximum (EC_{50}). [d] B_{max} is the maximum level of binding at saturating [PCB]. [e] Above solubility limit.

activation of the RyR1 channel complex may be useful for risk assessment of environmental mixtures containing ortho-substituted PCB congeners that have low, or lack, Ah receptor activity.

Figure 3a shows the dose−response curves of 4-OH,2,3,6,-2′,3′,6′-hexachloro-biphenyl (4-OH-PCB136), PCB136 (2,3,6,-2′,3′,6′-hexchlorobiphenyl), and PCB176 (2,3,4,6,2′,3′,6′-heptachlorobiphenyl or 4-Cl-PCB136). The metabolite 4-OH-PCB136 is a potential metabolic product of PCB136 produced by a cytochrome P450-catalyzed oxidation. The addition of a hydroxyl group at the para-position reduces EC_{2x} potency toward RyR1 activation seven-fold as compared to the parent compound (Figure 3a). Comparing the activities of PCB136 and PCB176 in Figure 3a shows that although a *para*-chloro substitution lowers the maximum efficacy (maximum level of RyR1 activation) of this congener relative to PCB136, the *para*-chloro substitution maintains a higher potency toward activating RyR1 than its *para*-hydroxy derivative. This may be due to the difference in size and/or the electronegativity of the substitutients. A hydroxyl group on a phenyl ring is generally considered to be highly acidic (electron withdrawing), whereas a chloro moiety at the same position on the phenyl ring is considered a deactivating group (electron donating).

Conversely, Figure 3b and Table 1 show that the 4′-OH,2,4,6-Cl derivative (4′OH-PCB30) is significantly more active than the parent PCB30 (2,4,6-Cl). Thus, a *para*-OH group on the phenyl ring that carries no other deactivating substitution confers potency and efficacy toward activating RyR1. Two possible mechanisms could be responsible. First, the acidic property of the lone phenyl-OH substituent is likely to contribute hydrogen-bonding potential to stabilize interactions with the receptor site. Alternatively, if the hydroxyl group were partially or wholly ionized, then electrostatic interactions would be expected to stabilize the PCB−RyR1 complex. In support of this interpretation, the di-OH derivative of PCB30, 3′,4′-di-OH,2,4,6-PCB, was found to possess lower potency but similar efficacy to PCB30. The presence of two adjacent −OH moieties would be expected to promote intramolecular hydrogen bonding and could preclude stabilizing interactions with RyR1 that impacts the apparent affinity and efficacy for channel activation. Comparing the relative activities of PCB30 and PCB75 (2,4,6,4′-tetrachlorobiphenyl), the additional *para*-Cl substitution com-

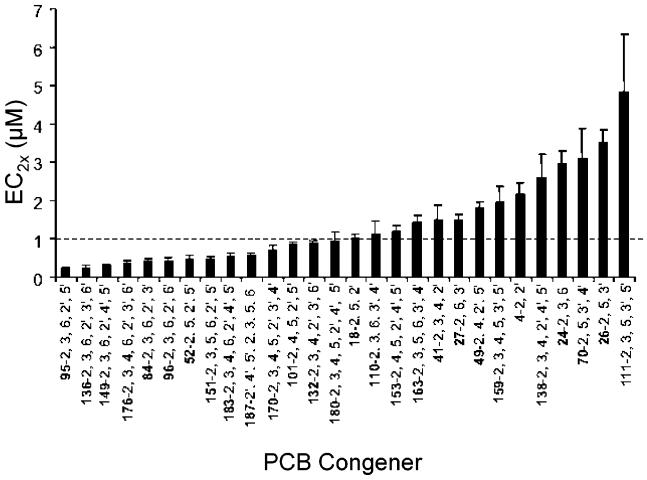

Figure 1. Rank potency of 28 PCB congeners possessing the highest potency toward activating RyR1. For each PCB congener, the concentration required to double the RyR1 channel activity (EC_{2x}; $p \leq 0.05$) was derived from complete dose−response curves from $n \geq 4$ independent experiments as described in the Experimental Procedures. Table 1 summarizes parameters obtained for all of the congeners tested.

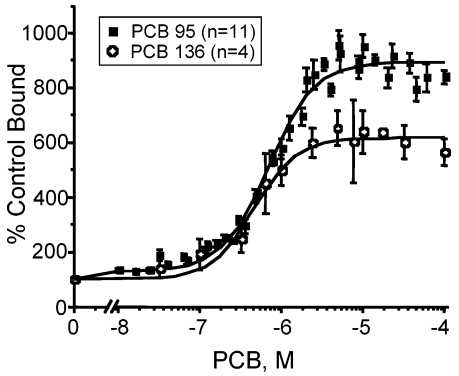

Figure 2. PCB95 and PCB136 show high potency and efficacy toward activation of RyR1. Equilibrium binding of [^{3}H]Ry to RyR1 was performed in the absence and presence of PCB95 or PCB136 (10 nM to 100 μM) as described in the Experimental Procedures. The binding experiment was repeated with at least two different tissue preparations, and averaged results are shown in the figure for PCB95 ($n = 11$) and PCB136 ($n = 4$). Data were plotted as % of baseline specific binding measured in the absence of PCB (i.e., % control). The data shown are the averages of all of the measurements normalized to their respective control binding (in the presence of equivalent amount of DMSO) in each measurement and the standard error of the replicates. The parameters EC_{2x}, EC_{50}, and B_{max} are summarized in Table 1.

pletely eliminates activity toward RyR1. Thus, a complete lack of activity observed here with PCB75 is likely due to the di-*para*-chloro substitution. In general, PCB structures possessing 4,4′-Cl possess lower activity toward RyR1, regardless of the presence of one or more ortho substitutions (e.g., PCB66, -75, -123, -138, -153, and -157; Table 1). To further understand the influence of hydroxylation on the activity of PCBs toward RyR1 activation, the SAR was extended to hydroxylated derivatives of PCB9 (2,5-Cl) and PCB18 (2,5,2′-Cl). Although both di-OH derivatives of PCB9 were less active, the presence of *meta*/*para*-hydroxyls imparted lower potency but similar efficacy toward activating RyR1 as compared to the parent structure, whereas the corresponding ortho/meta derivative possessed significantly lower potency and efficacy (Figure 4a). Interestingly, PCB9 derivatives possessing either 3′-OH or 4′-OH substitution exhibited significantly higher potency and efficacy than the parent (Figure 4b). Collectively, these results underscore the importance of para substituents in conferring optimal RyR1 activity as was suggested earlier (*37*). Importantly, these results

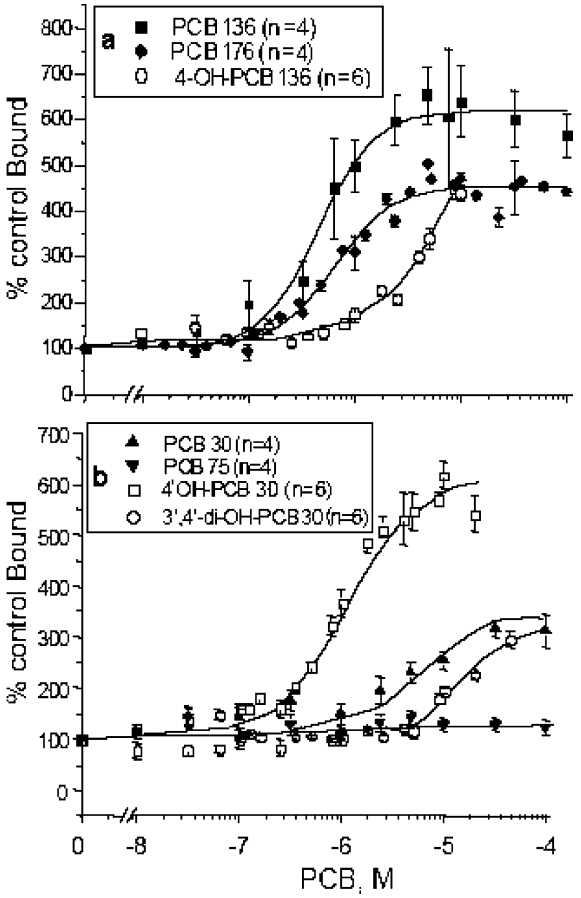

Figure 3. Dose−response curves of hydroxylated metabolites of PCB136 and PCB30 as compared to their probable parent structures. Equilibrium binding of [^{3}H]Ry to RyR1 was performed as described in the Experimental Procedures in the presence of (a) PCB136, PCB176, or 4-OH,2,3,6,2′,3′,6′-PCB (10 nM to 100 μM). Part b shows the dose−response relationship between PCB30, PCB75, 4′-OH,2,4,6-PCB, and 3′,4′-di-OH,2,4,6-PCB and [^{3}H]Ry binding to RyR1. EC_{2x}, EC_{50}, and B_{max} are summarized in Table 1.

also suggest that metabolic hydroxylation of certain PCBs could yield structures possessing higher activity toward microsomal Ca^{2+} channels. In this regard, phenolic PCBs and $MeSO_2$-CBs have been shown to persist in blood or tissues of laboratory animals (*57*, *58*) as well as in humans (*59*).

Figure 5a,b shows the dose−response curves for methyl-sulfonyl-PCBs and their respective potential parent structures. The presence of methyl-sulfonyl substitution disfavors the activity of the metabolites toward activation of RyR1. These findings further show that the presence of a bulky group in the para-position decreases the efficacy of the PCB structure toward activation of RyR1, further establishing that substitution at the para-position is essential for PCB binding to RyR1. Interestingly, the presence of either a *para*-Cl or a methyl-sulfonyl group destabilizes the binding of PCBs to RyR1.

FKBP12 Is Required for PCB-Induced Release of Microsomal Ca^{2+}. The activity of several PCB congeners to induce Ca^{2+} efflux from isolated microsomal vesicles was studied in order to determine if the SAR identified in radioligand binding studies with [^{3}H]Ry quantitatively predicts the ability of PCBs to dysregulate Ca^{2+} transport properties across the ER/SR membrane. Consistent with published results with PCB95, PCB187 induces a net Ca^{2+} efflux from Ca^{2+}-loaded vesicles (Figure 6A). The PCB-induced Ca^{2+} efflux could be completely blocked when the microsomal vesicles were preincubated with ruthenium red, a selective RyR blocker (Figure 6A, trace b).

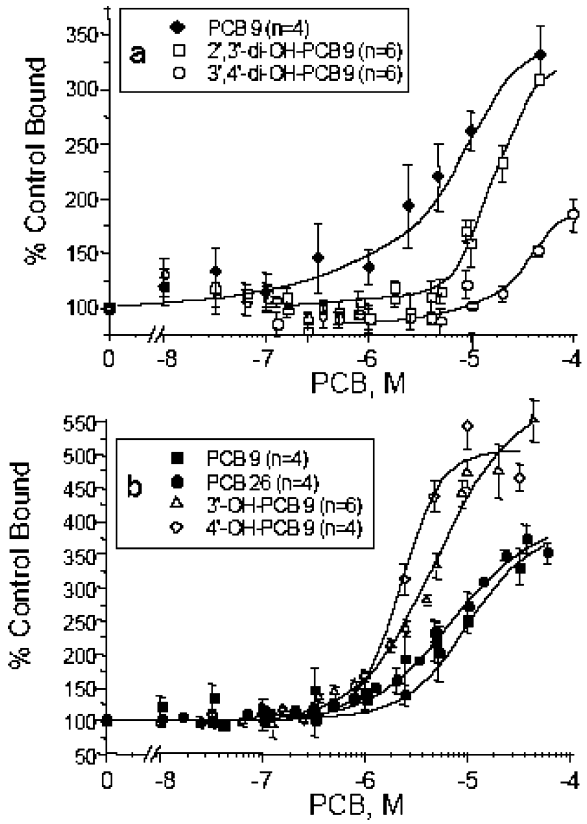

Figure 4. Dose−response curves of PCB9 and hydroxylated derivatives. Part a shows the dose−response curves of PCB9, PCB26, and metabolites 2′,3′-di-OH,2,5-PCB and 3′,4′-di-OH,2,5-PCB, and part b shows the curves from 3′-OH,2,5-PCB and 4′-OH,2,5-PCB. All measurements were repeated with at least two independent tissue preparations. The data shown are the averages of all of the measurements normalized to their respective control binding (in the presence of equivalent amount of DMSO) in each measurement and the standard error of the replicates. EC_{2x}, EC_{50}, and B_{max} are summarized in Table 1.

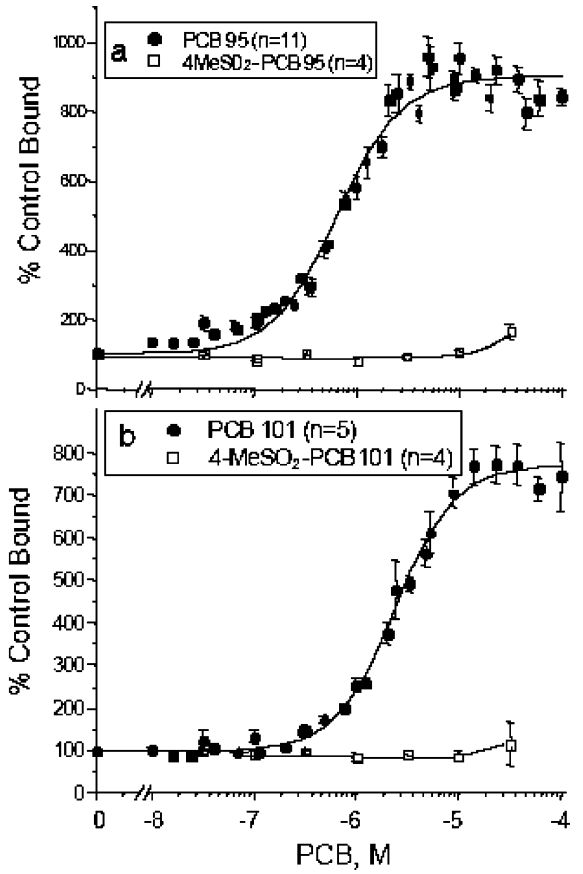

Figure 5. Dose−response curves of selected methyl-sulfonyl-PCB metabolites and their parent structures. Part a shows the dose−response curves of PCB95 and 4′-methyl-sulfonyl-PCB95. In contrast to highly active PCB95, its 4′-methyl-sulfonyl-metabolite does not activate RyR1. Part b shows the dose−response curves of PCB101 and 4-methyl-sulfonly-PCB101. Part c shows the dose−response curves of PCB149, 4-methyl-sulfonyl-PCB149, and 3′-methyl-sulfonyl-PCB149. All measurements were repeated *n* times (as shown in the figure) with at least two independent tissue preparations. The data shown are the averages of all of the measurements normalized to their respective control binding (in the presence of equivalent amount of DMSO) in each measurement and the standard error of the replicates.

Immunophilin FKBP12 tightly associates with RyR1 thereby regulating both the gating and the kinetic properties of the channel (*60*). Immunosuppressants such as FK506 or rapamycin destabilize the RyR1−FKBP12 interaction thereby promoting dissociation of the complex. We have previously shown that dissociation of the RyR1−FKBP12 complex completely and selectively eliminates RyR1 Ca^{2+} channel responses to PCB95 (*42*). We further explored if an intact RyR1−FKBP12 complex was a general requirement for the actions of noncoplanar PCBs exhibiting potent RyR1 activity. Microsomal vesicles were pretreated for 2 min with rapamycin to dissociate FKBP12 from RyR1, and subsequent challenge with the tri-*ortho*-heptachlorobiphenyl PCB187 (7.5 μM) significantly diminished its ability to mobilize microsomal Ca^{2+} (Figure 6B, compare traces a and b). Elimination of PCB187-induced Ca^{2+} release by rapamycin was not the result of a general block of RyR1 channel function, as it is with ruthenium red, since subsequent addition of caffeine induced rapid release of Ca^{2+} from the microsomes (Figure 6B, trace b). The same requirement for an intact FKBP12−RyR1 complex was observed with each of 12 active PCB congeners indicating a common mechanism requiring an immunophilin-regulated Ca^{2+} release channel (not shown). We further tested the correlation between the potency of 14 PCBs to release Ca^{2+} from actively loaded microsomes with their ability to enhance the amount of high-affinity [^{3}H]Ry binding.

Correlation between PCB-Sensitized [^{3}H]Ry Binding and ER/SR Ca^{2+} Release. Figure 7 shows the correlation between the initial rate of PCB-induced Ca^{2+} efflux (normalized to PCB95) and the EC_{2x} calculated from [^{3}H]Ry binding experiments. These results indicate that results from the EC_{2x} potency parameter obtained from [^{3}H]Ry binding analysis provide an excellent prediction of microsomal Ca^{2+} dysregulation (r^2 = 0.87).

Application of RyR1-Based SAR To Assess Activity in Environmental Mixtures. Well-characterized extracts of environmental media collected from a PCB-contaminated landfill were tested for their activity toward sensitizing activation of RyR1 channels. On the basis of the average molecular weight of the PCBs contained in each mixture (Table 2), each extract was found to possess a distinct structure−activity profile in terms of EC_{50} and maximum level of RyR1 activation (Figure 8). Intuitively, it was hypothesized that the air extract (vapor phase PCBs) would have the greatest potency and efficacy. This was because, in addition to increased sensitization of RyR1, ortho-rich/para-poor PCB congeners have higher vapor pressures than ortho-poor/para-rich congeners and tend to achieve higher relative levels in air (*1*, *61*, *62*). Indeed, the dust contained a greater proportion of the less active higher chlorinated biphenyls (66, 138, 153, 170) and less of the active 18 and 52 (Table 3); however, dust also contained nearly twice the proportion of active more highly chlorinated congeners (84 + 95 + 101 + 110 + 132 + 136 + 149 + 151 = 16.7%) as compared to air

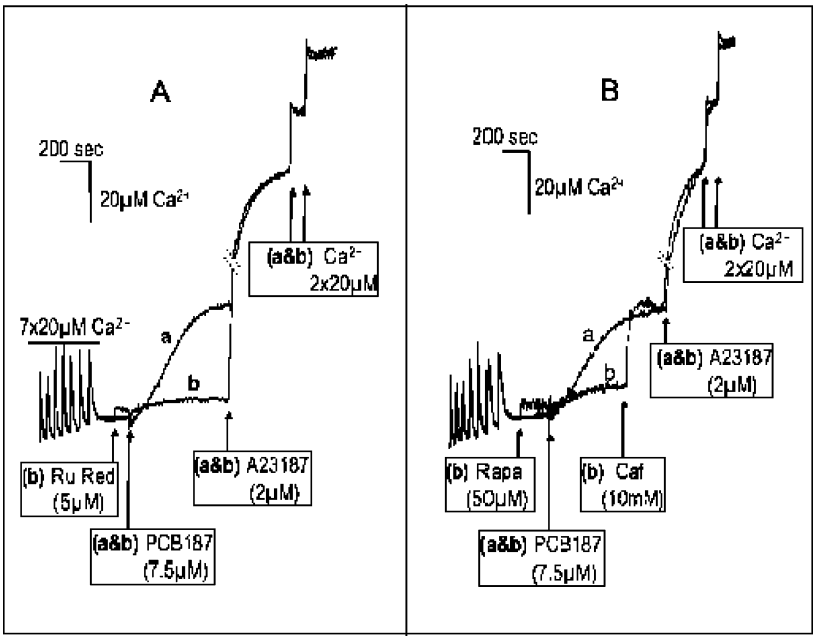

Figure 6. PCB-induced Ca^{2+} efflux through RyR1/FKBP12-mediated pathway. Active Ca^{2+} efflux was measured as described in the Experimental Procedures. (Parts A and B, traces labeled a) PCB187 (7.5 μM) induces Ca^{2+} efflux from the actively loaded microsomal vesicles. (Part A, trace b) The PCB187-induced Ca^{2+} efflux was completely blocked by pretreating the vesicles with 5 μM ruthenium red, which selectively blocked RyR1 channels. (Part B, trace b) Preincubation of the vesicles with 50 μM rapamycin, which binds to FKBP12 and dissociates FKBP12 from the RyR1 complex, blocked the PCB187-induced Ca^{2+} efflux selectively. Subsequent addition of 10 mM caffeine induced Ca^{2+} release, showing that the functional integrity of the ryanodine sensitive Ca^{2+} release channel was preserved after rapamycin treatment. The experiment shown is the respective of three replicates with identical results.

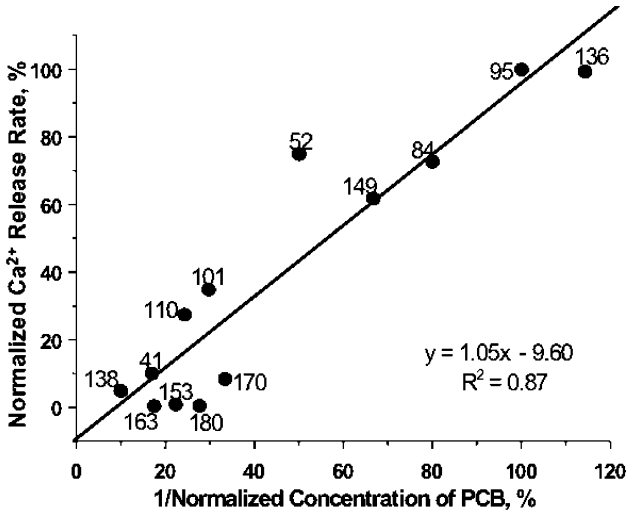

Figure 7. Correlation between efficacy of selected PCB congeners on [^{3}H]Ry binding to RyR1 and rate of Ca^{2+} efflux through RyR1. The rate of initial Ca^{2+} release induced by various PCB congeners was determined and normalized to that of PCB95 at 7.5 μM. The normalized Ca^{2+} efflux rate of selected PCB congeners is plotted against the normalized values of effective concentration that doubles the baseline [^{3}H]Ry binding level of the respective congeners. A direct correlation between the Ca^{2+} release rate and the relative EC_{2x} obtained in [^{3}H]Ry binding experiments is observed (correlation coefficient $R^2 = 0.87$), suggesting a selective and specific interaction between PCBs and RyR1 to sensitize the release of Ca^{2+} from microsomal vesicles. The data shown are the averages of 2−3 replicates.

(9.1%) and soil (8.7%) extracts (Table 3). The lower activity soil contained the lowest proportion of higher chlorinated RyR1-active congeners and, as compared to air, slightly lower proportions of the highly active PCB18 and -52.

The unique congener distribution patterns were explained by microbial dechlorination in the perpetually wet soils, volatilization from the wet soils, and trapping of the volatile congeners

Table 2. Major Chlorinated Aromatic Compounds and TCDD Equivalencies in Refined Extracts of Air, Dust, and Soil from a PCB-Contaminated Landfill

	concentration in extract (mg/L)		
compounds	air extract	dust extract	soil extract
total PCB	4600	20660	46890
monoCBs + DiCBs (%)	2.5%	0.2%	1.4%
triCBs	50.8%	22.5%	44.4%
tetraCBs	34.4%	35.0%	37.7%
pentaCBs	10.1%	25.3%	12.1%
hexaCBs	2.1%	13.8%	4.2%
hepta- to decaCBs	0.1%	3.3%	1.4%
average MW	277	308	284
PCB TCDD EQ	0.1	1.5	1.4
total PCDDs	<0.01	11.4	47.3
PCDD TCDD EQ	0	0.00006	0.0007
total PCDFs	23.1	250	762
PCDF TCDD EQ	0.004	0.012	0.032
4,4′-**DDE**	<0.1	176	125

in the dryer superficial dust layers (*49*). PCB28 (2,4,4′-triCB) dominated in all three extracts (Table 3). Although it was not tested in this study, the structure would indicate low RyR1 activity so that it would serve as a diluent, especially in the air and soil extracts. These differences in PCB composition help to explain the divergent activities of the three environmental extracts from the same location.

Predicting Neurotoxic Risk of PCBs Based on RyR1 Channel Activity. In most mammalian cells, all three genetic isoforms of RyRs (RyR1, RyR2, and RyR3) function as Ca^{2+}-induced Ca^{2+} release (CICR) channels. Therefore, signals mediated by activation of CICR are typically coordinated with IP_3R-mediated releases of Ca^{2+} from SR/ER stores and conformational coupling to cell surface channels. The three genetic isoforms of RyR are widely expressed in all tissues and in a developmentally and functionally regulated manner. Considering the neurodevelopmental toxicity attributed to PCBs (*27*), the role of RyRs in the central nervous system in the formation and elongation of dendritic spines and inhibitory presynaptic

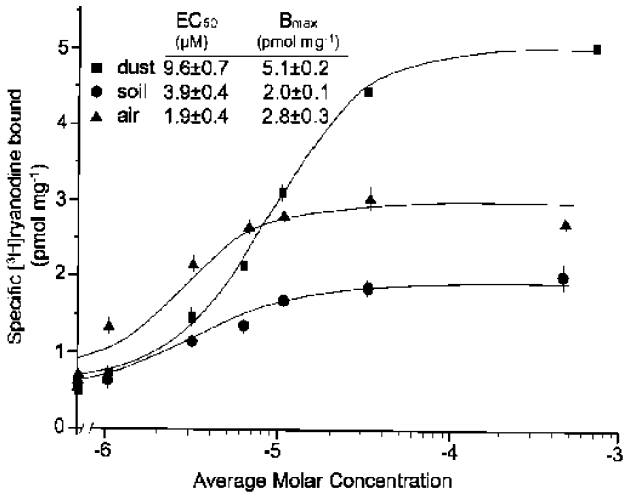

Figure 8. Detection of RyR1-active PCBs in extracts from a National Priorities List Landfill. Total PCBs were extracted from subsurface soil (●), surface dust (■), and air (▲) as described in the Experimental Procedures. Partial congener profiles for each sample are shown in Tables 2 and 3. Parallel samples were extracted from an area remote from point sources of PCBs and lacked detectable activation relative to controls.

Table 3. Percent Contribution of Major and/or RyR1-Active PCB Congeners in the Environmental Extracts

		relative amount in extract (% total)[a]		
congener	RyR1 max relative to PCB 95	air extract	dust extract	soil extract
18	0.77	5.8	0.8	4.7
28	not done	17.4	8.7	15.5
49	0.45	4.0	2.9	3.9
52	0.53	6.9	4.5	6.6
66	0.22	3.2	4.8	4.2
70	0.29	2.7	4.1	3.5
84	0.82	1.1	1.2	0.4
95	1.00	2.1	2.3	2.1
101	0.52	2.6	4.2	2.2
110	0.61	2.2	5.3	2.2
132	0.51	0.1	0.6	0.2
136	0.60	0.2	0.4	0.2
138	0.33	0.4	3.3	1.2
149	1.03	0.6	2.1	1.2
151	0.72	0.2	0.5	0.2
153	0.44	0.5	2.2	0.9
170	0.33	0.01	0.5	0.2
180	0.41	0.01	0.7	0.5
183	0.33	0.01	0.2	0.1

[a] Average of two separate determinations by independent analytical laboratories (*1*).

terminals (*63*, *64*), neuroplasticity (*65*), and spatial learning (*66*), these channels are likely to represent a relevant receptor target for PCB toxicity (*39*). Noncoplanar PCB interactions with RyR complexes have also been shown to enhance NMDA-mediated excitotoxicity and apoptosis of neurons in vitro (*33*, *41*). To further understand the effects of PCBs on disruption of Ca^{2+} signaling, we performed the first detailed SAR between 34 PCB congeners and 12 PCB metabolites toward activation of RyR1. The dose−response relationships between selected PCB congeners and activity of RyR1 were determined by a radioligand binding assay using [^{3}H]Ry (the conformation selective probe specific for ryanodine receptors) and microsomal preparations enriched in RyR1 isolated from rabbit fast-twitch skeletal muscle. Our results have revealed that the *ortho*- and *meta*-chloro substitutions on the biphenyl structure of PCBs are the most important determinants of efficacy toward activation of RyR1.

Certain dioxin-like PCB congeners have been shown to cause toxicity similar to 2,3,7,8-TCDD, the most potent congener in the PCDD family. The toxicity responses include chloracne, wasting syndrome, increase incidence of soft tissue sarcomas, immunotoxicity, reproductive toxicity, developmental toxicity, disruption of endocrine pathways, hepatotoxicity, and thymic and splenic atrophy. These toxic responses might be mediated through a common pathway, which involves binding of HAHs to the Ah receptor. Among the structures examined, 2,3,7,8-TCDD has been shown to have the highest affinity toward the Ah receptor (*67*). As a result, the relative toxicities of dioxin-like PCB, PCDD, and PCDF congeners have been ranked according to their relative binding affinity to the Ah receptor. By contrast, PCBs that lack any *ortho*-chlorine substitution have been found to lack RyR1 activity (Pessah, unpublished data). Conversely, PCB95, which possesses high RyR1 and RyR2 activity (*35*, *38*), lacks Ah receptor activity (M. Denison, personal communication). The biological activities of coplanar and noncoplanar PCBs can therefore be distinguished on the basis of two distinct mechanisms, which may predict most of their toxicological effects.

The potential toxicity of coplanar PCBs is assessed based on their activity toward the Ah receptor, with the most potent agonist, 2,3,7,8-TCDD, assigned a TCDD equivalence factor (TEF) of 1.0 (*2*). In 1997, the World Health Organization held a meeting to derive consensus on TEFs for PCDDs, PCDFs, and dioxin-like PCBs for risk assessment on human, fish, and wildlife health (*68*). The TEF concept is based on a major assumption: At low dose, the TCDD equivalent (TEQ) concentration of HAHs in mixtures is additive, with no synergism or antagonism among the individual congeners. Several limitations on the TEF concepts have been identified (*69*). In most of the biological/environmental samples, relatively low concentrations of dioxin-like PCB congeners have been found, as compared to nondioxin-like PCB congeners with low TEFs. As a result of partial agonistic or antagonistic properties of certain PCB congeners, nonadditive interactions of PCB mixtures have been reported. TEF values are based on in vivo and in vitro studies in biotic systems. The influence in physiochemical factors that governed actual uptake of these chemicals from abiotic to biotic systems is not determined. More importantly, because of the fact that TEFs are derived from Ah receptor-mediated mechanisms, modulations through non-Ah receptor-mediated mechanisms are not considered. Conclusions drawn from the meeting reaffirmed that the TEF concept is the most plausible and feasible approach for risk assessment of dioxin-like HAHs. However, the uses of TEF on nondioxin-like PCB congeners, which are di-ortho or higher ortho-substituted, are excluded due to insufficient in vivo evidence (*68*). Because ortho-substituted PCB congeners collectively represent a significant component of PCB mixtures found in environmental samples and biological tissues (e.g., *56*), it is urgent to develop new approaches for risk assessment of ortho-substituted PCB congeners based on critical mechanisms responsible for initiating dysfunction leading to toxicity.

Conclusion. We propose the use of the SARs between ortho-substituted PCBs (and metabolites) and activation of RyR1 and its two alternate type 2 and type 3 isoforms, to derive alternative TEFs for the nondioxin-like PCBs. Our results thus far indicate purely additive effects when two or more noncoplanar PCBs are tested in the [^{3}H]Ry-binding assay. Additivity also appears to occur with complex mixtures (*56*). Importantly, coplanar PCBs such as PCB126 do not antagonize the actions of noncoplanar PCBs. The RyR bioassay does not have the same

limitations known to exist with TEFs based on AhR activity. With the application of the alternative TEFs, we can now further extend the risk assessment of nondioxin-like HAHs in the environmental complex mixtures.

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References

(1) Hansen, L. G. (1998) Stepping backward to improve assessment of PCB congener toxicities. *Environ. Health Perspect. 106* (Suppl. 1), 171−189.

(2) Safe, S., Astroff, B., Harris, M., Zacharewski, T., Dickerson, R., Romkes, M., and Biegel, L. (1991) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related compounds as antioestrogens: Characterization and mechanism of action. *Pharmacol. Toxicol. 69*, 400−409.

(3) Safe, S. H. (1994) Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit. Rev. Toxicol. 24*, 87−149.

(4) Safe, S. (2001) Molecular biology of the Ah receptor and its role in carcinogenesis. *Toxicol. Lett. 120*, 1−7.

(5) Frame, G. M., II (1999) Appropriate characterization of PCB mixtures appearing as synthetic byproducts. *J. High Resolut. Chromatogr. 22*, 73.

(6) Frame, G. M. C., Jack, W., and Bowadt, S. S. (1996) Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative, congener-specific analysis. *J. High Resolut. Chromatogr. 19*, 657−668.

(7) Humphrey, H. E., Joseph, J. C., Pandya, J. R., Sweeney, A. M., Gasior, D. M., McCaffrey, R. J., and Schantz, S. L. (2000) PCB congener profile in the serum of humans consuming Great Lakes fish. *Environ. Health Perspect. 108*, 167−172.

(8) Cousins, I. T., Hartlieb, N., Teichmann, C., and Jones, K. C. (1996) Volatilization of polychlorinated biphenyls from sludge-amended soils. *Organohalogen Compd. 28*, 58−63.

(9) Hornbuckle, K. C., Clyde, W., Pearson, R. F., Swackhamer, D. L., and Eisenreich, S. J. (1995) Assessing annual water−air fluxes of polychlorinated biphenyls in Lake Michigan. *Environ. Sci. Technol. 29*, 869−877.

(10) Miller, S. M., Mark, L., DePinto, J. V., and Hornbuckle, K. C. (2001) Results from the Lake Michigan mass balance study: Concentrations and fluxes of atmospheric polychlorinated biphenyls and trans-Nonachlor. *Environ. Sci. Technol. 35*, 278−285.

(11) Wethington, D. M., III, and Hornbuckle, K. C. (2004) Milwaukee, WI, as a source of atmospheric PCBs to Lake Michigan. *Environ. Sci. Technol. 39*, 57−63.

(12) Porte, C., and Albaiges, J. (1994) Bioaccumulation patterns of hydrocarbons and polychlorinated biphenyls in bivalves, crustaceans, and fishes. *Arch. Environ. Contam. Toxicol. 26*, 273−281.

(13) Gerstenberger, S. L., Gallinet, M. P., and Dellinger, J. A. (1997) Polychlorinated biphenyl congeners and selected organochlorines in Lake Superior fish, USA. *Environ. Toxicol. Chem. 16*, 2222−2228.

(14) Gerstenberger, S. L., and Dellinger, J. A. (2002) PCBs, mercury, and organochlorine concentrations in lake trout, walleye, and whitefish from selected tribal fisheries in the Upper Great Lakes region. *Environ. Toxicol. 17*, 513−519.

(15) Gerstenberger, S. L., Dellinger, J. A., and Hansen, L. G. (2000) Concentrations and frequencies of polychlorinated biphenyl congeners in a Native American population that consumes Great Lakes fish. *J. Toxicol. Clin. Toxicol. 38*, 729−746.

(16) Bedard, D. L. (2001) In *PCBs, Recent Advances in Environmental Toxicology and Health Effects* (Robertson, L. W., and Hansen, L. G., Eds.) pp 27−33, University Press of Kentucky, Lexington, KY.

(17) Bae, J., Peters-Golden, M., and Loch-Caruso, R. (1999) Stimulation of pregnant rat uterine contraction by the polychlorinated biphenyl (PCB) mixture aroclor 1242 may be mediated by arachidonic acid release through activation of phospholipase A2 enzymes. *J. Pharmacol. Exp. Ther. 289*, 1112−1120.

(18) Bae, J., Stuenkel, E. L., and Loch-Caruso, R. (1999) Stimulation of oscillatory uterine contraction by the PCB mixture Aroclor 1242 may involve increased $[Ca^{2+}]$i through voltage-operated calcium channels. *Toxicol. Appl. Pharmacol. 155*, 261−272.

(19) Fischer, L. J., Wagner, M. A., and Madhukar, B. V. (1999) Potential involvement of calcium, CaM kinase II, and MAP kinases in PCB-stimulated insulin release from RINm5F cells. *Toxicol. Appl. Pharmacol. 159*, 194−203.

(20) Olivero, J., and Ganey, P. E. (2000) Role of protein phosphorylation in activation of phospholipase A2 by the polychlorinated biphenyl mixture Aroclor 1242. *Toxicol. Appl. Pharmacol. 163*, 9−16.

(21) Garner, C. E., Burka, L. T., Etheridge, A. E., and Matthews, H. B. (2000) Catechol metabolites of polychlorinated biphenyls inhibit the catechol-O-methyltransferase-mediated metabolism of catechol estrogens. *Toxicol. Appl. Pharmacol. 162*, 115−123.

(22) Khan, M. A., Lichtensteiger, C. A., Faroon, O., Mumtaz, M., Schaeffer, D. J., and Hansen, L. G. (2002) The hypothalamo-pituitary-thyroid (HPT) axis: A target of nonpersistent ortho-substituted PCB congeners. *Toxicol. Sci. 65*, 52−61.

(23) Li, H., Boon, J. P., Lewis, W. E., van den Berg, M., Nyman, M., and Letcher, R. J. (2003) Hepatic microsomal cytochrome P450 enzyme activity in relation to in vitro metabolism/inhibition of polychlorinated biphenyls and testosterone in Baltic gray seal (Halichoerus grypus). *Environ. Toxicol. Chem. 22*, 636−644.

(24) Li, M. H., and Hansen, L. G. (1996) Enzyme induction and acute endocrine effects in prepubertal female rats receiving environmental PCB/PCDF/PCDD mixtures. *Environ. Health. Perspect. 104*, 712−722.

(25) Jacobson, J. L., and Jacobson, S. W. (1996) Intellectual impairment in children exposed to polychlorinated biphenyls in utero. *N. Engl. J. Med. 335*, 783−789.

(26) Schantz, S. L., Gasior, D. M., Polverejan, E., McCaffrey, R. J., Sweeney, A. M., Humphrey, H. E., and Gardiner, J. C. (2001) Impairments of memory and learning in older adults exposed to polychlorinated biphenyls via consumption of Great Lakes fish. *Environ. Health. Perspect. 109*, 605−611.

(27) Schantz, S. L., Widholm, J. J., and Rice, D. C. (2003) Effects of PCB exposure on neuropsychological function in children. *Environ. Health. Perspect. 111*, 357−576.

(28) Corrigan, F. M., Murray, L., Wyatt, C. L., and Shore, R. F. (1998) Diorthosubstituted polychlorinated biphenyls in caudate nucleus in Parkinson's disease. *Exp. Neurol. 150*, 339−342.

(29) Seegal, R. F., Bush, B., and Shain, W. (1990) Lightly chlorinated ortho-substituted PCB congeners decrease dopamine in nonhuman primate brain and in tissue culture. *Toxicol. Appl. Pharmacol. 106*, 136−144.

(30) Shain, W., Bush, B., and Seegal, R. (1991) Neurotoxicity of polychlorinated biphenyls: Structure−activity relationship of individual congeners. *Toxicol. Appl. Pharmacol. 111*, 33−42.

(31) Kodavanti, P. R., Shin, D. S., Tilson, H. A., and Harry, G. J. (1993) Comparative effects of two polychlorinated biphenyl congeners on calcium homeostasis in rat cerebellar granule cells. *Toxicol. Appl. Pharmacol. 123*, 97−106.

(32) Kodavanti, P. R., Ward, T. R., McKinney, J. D., and Tilson, H. A. (1995) Increased [^{3}H]phorbol ester binding in rat cerebellar granule cells by polychlorinated biphenyl mixtures and congeners: Structure−activity relationships. *Toxicol. Appl. Pharmacol. 130*, 140−148.

(33) Howard, A. S., Fitzpatrick, R., Pessah, I., Kostyniak, P., and Lein, P. J. (2003) Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor. *Toxicol. Appl. Pharmacol. 190*, 72−86.

(34) Schantz, S. L., Seo, B. W., Wong, P. W., and Pessah, I. N. (1997) Long-term effects of developmental exposure to 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) on locomotor activity, spatial learning and memory and brain ryanodine binding. *Neurotoxicology 18*, 457−467.

(35) Wong, P. W., Brackney, W. R., and Pessah, I. N. (1997) Ortho-substituted polychlorinated biphenyls alter microsomal calcium transport by direct interaction with ryanodine receptors of mammalian brain. *J. Biol. Chem. 272*, 15145−15153.

(36) Wong, P. W., Garcia, E. F., and Pessah, I. N. (2001) ortho-substituted PCB95 alters intracellular calcium signaling and causes cellular acidification in PC12 cells by an immunophilin-dependent mechanism. *J. Neurochem. 76*, 450−463.

(37) Wong, P. W., Joy, R. M., Albertson, T. E., Schantz, S. L., and Pessah, I. N. (1997) Ortho-substituted 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) alters rat hippocampal ryanodine receptors and neuroplasticity in vitro: Evidence for altered hippocampal function. *Neurotoxicology 18*, 443−456.

(38) Wong, P. W., and Pessah, I. N. (1996) Ortho-substituted polychlorinated biphenyls alter calcium regulation by a ryanodine receptor-mediated mechanism: Structural specificity toward skeletal- and cardiac-type microsomal calcium release channels. *Mol. Pharmacol. 49*, 740−751.

(39) Pessah, I. N., and Wong, P. W. (2001) In *Recent Advances in Environmental Toxicology and Health Effects* (Robertson, L. W.,

Hansen, L. G., Eds.) pp 179−184, University Press of Kentucky, Lexington, KY.
(40) Inglefield, J. R., Mundy, W. R., and Shafer, T. J. (2001) Inositol 1,4,5-triphosphate receptor-sensitive Ca(2+) release, store-operated Ca(2+) entry, and cAMP responsive element binding protein phosphorylation in developing cortical cells following exposure to polychlorinated biphenyls. *J. Pharmacol. Exp. Ther. 297*, 762−773.
(41) Gafni, J., Wong, P. W., and Pessah, I. N. (2004) Noncoplanar 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) amplifies ionotropic glutamate receptor signaling in embryonic cerebellar granule neurons by a mechanism involving ryanodine receptors. *Toxicol. Sci. 77*, 72−82.
(42) Wong, P. W., and Pessah, I. N. (1997) Noncoplanar PCB 95 alters microsomal calcium transport by an immunophilin FKBP12-dependent mechanism. *Mol. Pharmacol. 51*, 693−702.
(43) Patterson, R. L., Boehning, D., and Snyder, S. H. (2004) Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu. Rev. Biochem. 73*, 437−465.
(44) Guatimosim, S., Dilly, K., Santana, L. F., Saleet Jafri, M., Sobie, E. A., and Lederer, W. J. (2002) Local Ca^{2+} signaling and EC coupling in heart: Ca^{2+} sparks and the regulation of the $[Ca^{2+}]$(i) transient. *J. Mol. Cell Cardiol. 34*, 941−950.
(45) Augustine, G. J., Santamaria, F., and Tanaka, K. (2003) Local calcium signaling in neurons. *Neuron 40*, 331−346.
(46) Pessah, I. N., Kim, K. H., and Feng, W. (2002) Redox sensing properties of the ryanodine receptor complex. *Front. Biosci. 7*, a72−a79.
(47) Gronert, G. A., Pessah, I. N., Muldoon, S. M., and Tautz, T. J. (2004) *Malignant Hyperthermia*, 6th ed. (Miller, R. D., Ed.) Churchill Livingstone, Philadelphia, PA.
(48) Wehrens, X. H., and Marks, A. R. (2004) Sudden unexplained death caused by cardiac ryanodine receptor (RyR2) mutations. *Mayo Clin. Proc. 79*, 1367−1371.
(49) Hansen, L., Greene, D., Cochran, J., Vermette, S., and Bush, B. (1997) Chlorobiphenyl (PCB) composition of extracts of subsurface soil, superficial dust and air from a contaminated landfill. *Fresenius J. Anal. Chem. 357*, 442−448.
(50) Hansen, L. G., and O'Keefe, P. W. (1996) Polychlorinated dibenzofurans and dibenzo-p-dioxins in subsurface soil, superficial dust, and air extracts from a contaminated landfill. *Arch. Environ. Contam. Toxicol. 31*, 271−276.
(51) Li, M. H., and Hansen, L. G. (1996) Responses of prepubertal female rats to environmental PCBs with high and low dioxin equivalencies. *Fundam. Appl. Toxicol. 33*, 282−293.
(52) Pessah, I. N., Stambuk, R. A., and Casida, J. E. (1987) Ca2+-activated ryanodine binding: Mechanisms of sensitivity and intensity modulation by Mg2+, caffeine, and adenine nucleotides. *Mol. Pharmacol. 31*, 232−238.
(53) Pessah, I. N., Durie, E. L., Schiedt, M. J., and Zimanyi, I. (1990) Anthraquinone-sensitized Ca2+ release channel from rat cardiac sarcoplasmic reticulum: Possible receptor-mediated mechanism of doxorubicin cardiomyopathy. *Mol. Pharmacol. 37*, 503−514.
(54) Lehmler, H. J., Robertson, L. W., and Parkin, S. (2005) 2,2′,3,3′,6-Pentachlorobiphenyl (PCB 84). *Acta Crystallogr., Sect. E: Struct. Rep. Online 61*, 3025−3026.
(55) Mack, M. M., Zimanyi, I., and Pessah, I. N. (1992) Discrimination of multiple binding sites for antagonists of the calcium release channel complex of skeletal and cardiac sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther. 262*, 1028−1037.
(56) Kostyniak, P. J., Hansen, L. G., Widholm, J. J., Fitzpatrick, R. D., Olson, J. R., Helferich, J. L., Kim, K. H., Sable, H. J., Seegal, R. F., Pessah, I. N., and Schantz, S. L. (2005) Formulation and characterization of an experimental PCB mixture designed to mimic human exposure from contaminated fish. *Toxicol. Sci.* **2005**, *88*, 400−411.
(57) Bergman, A., Klasson-Wehler, E., and Kuroki, H. (1994) Selective retention of hydroxylated PCB metabolites in blood. *Environ. Health Perspect. 102*, 464−469.
(58) Haraguchi, K., Kato, Y., Koga, N., and Degawa, M. (2004) Metabolism of polychlorinated biphenyls by Gunn rats: Identification and serum retention of catechol metabolites. *Chem. Res. Toxicol. 17*, 1684−1691.
(59) Sandau, C. D., Ayotte, P., Dewailly, E., Duffe, J., and Norstrom, R. J. (2000) Analysis of hydroxylated metabolites of PCBs (OH-PCBs) and other chlorinated phenolic compounds in whole blood from Canadian Inuit. *Environ. Health. Perspect. 108*, 611−616.
(60) Meissner, G. (2002) Regulation of mammalian ryanodine receptors. *Front. Biosci. 7*, d2072−d2080.
(61) Hermanson, M., and Hites, R. (1989) Long-term measurements of atmospheric polychlorinated biphenyls in the vicinity of superfund dumps. *Environ. Sci. Technol. 23*, 1253−1258.
(62) Hornbuckle, K., Achman, D., and Eisenreich, S. (1993) Over-water and over-land polychlorinated biphenyls in Green Bay, Lake Michigan. *Environ. Sci. Technol. 27*, 87−98.
(63) Korkotian, E., and Segal, M. (1999) Release of calcium from stores alters the morphology of dendritic spines in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A. 96*, 12068−12072.
(64) Conti, R., Tan, Y. P., and Llano, I. (2004) Action potential-evoked and ryanodine-sensitive spontaneous Ca2+ transients at the presynaptic terminal of a developing CNS inhibitory synapse. *J. Neurosci. 24*, 6946−6957.
(65) Unni, V. K., Zakharenko, S. S., Zablow, L., DeCostanzo, A. J., and Siegelbaum, S. A. (2004) Calcium release from presynaptic ryanodine-sensitive stores is required for long-term depression at hippocampal CA3-CA3 pyramidal neuron synapses. *J. Neurosci. 24*, 9612−9622.
(66) Zhao, W., Meiri, N., Xu, H., Cavallaro, S., Quattrone, A., Zhang, L., and Alkon, D. L. (2000) Spatial learning induced changes in expression of the ryanodine type II receptor in the rat hippocampus. *FASEB J. 14*, 290−300.
(67) Denison, M. S., and Nagy, S. R. (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol. 43*, 309−334.
(68) Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect. 106*, 775−792.
(69) Safe, S. H., Pallaroni, L., Yoon, K., Gaido, K., Ross, S., and McDonnell, D. (2002) Problems for risk assessment of endocrine-active estrogenic compounds. *Environ. Health Perspect. 110* (Suppl. 6), 925−929.

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